Oestrogen receptor α and β , androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep

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Abstract

A first step to elucidating the roles that steroids may play in the processes of ovarian development and early follicular growth is to identify the cell types that are likely to be receptive to steroids. Thus, cell types expressing receptors for oestrogen (α and β form; ER α and ER β respectively), androgen (AR) and progesterone (PR) were determined by *in situ* hybridisation and immunohistochemistry in ovine ovarian tissues collected during ovarian development and follicular formation (days 26–75 of fetal life) as well as during the early stages of follicular growth. Expression of ER β was observed early during ovarian development and continued to be expressed throughout follicular formation and also during the early stages of follicular growth. ER β was identified in germ cells as well as in the granulosa cells. At the large pre-antral stage of follicular growth, expression of ER α was also consistently observed in granulosa cells. AR was first consistently observed at day 55 of fetal life in stroma cells throughout the ovary. Within the follicle, expression was observed in granulosa and thecal cells from the type-2 to -3 stage of follicular growth. PR mRNA did not appear to be expressed during ovarian development (days 26–75 of gestation). However, PR (mRNA and protein) was observed in the theca of type-3 (small pre-antral) and larger follicles, with mRNA – but not protein – observed in granulosa cells of some type-4 and 5 follicles. Expression of ER β , ER α and AR, as well as PR, was also observed in the surface epithelium and ovarian stroma of the fetal, neonatal and adult ovary. Thus, in sheep, steroid hormones have the potential to regulate the function of a number of different ovarian cell types during development, follicular formation and early follicular growth.

Reproduction (2006) **131** 81–92

Introduction

Steroid hormones are known to play key roles in regulating ovarian function in mammals. In addition to the central role they play in the communication between the ovary and pituitary, steroids are also thought to act as intra-ovarian regulators although their roles are likely to differ between species. In mice, the ovary does not appear to produce steroids during fetal life (Greco & Payne 1994) and, when examined, mice lacking steroid receptors appeared to undergo normal ovarian development (Lydon *et al.* 1996, Couse & Korach 1999). However, in many other species the developing ovary is steroidogenically active (Mauleon *et al.* 1977, Weniger 1990, George & Wilson 1994, Lun *et al.* 1998). Moreover, in monkeys and sheep, prenatal/perinatal exposure to steroids during gonadal development and follicular formation leads to an altered ovarian morphology with a large number of cystic follicles (Abbott *et al.* 2002). Thus, in some species at

least, steroids may play a key role during gonadal development.

A critical role for steroids in regulating follicular growth has also been shown with the development of abnormal ovarian phenotypes associated with reduced fertility in mice lacking steroid receptors (Lydon *et al.* 1996, Drummond *et al.* 2002, Yeh *et al.* 2002). Some of these effects are related to changes in other hormones regulated by steroids – such as gonadotrophins – or follicular processes occurring late during follicular development; however, there is also growing evidence supporting a direct intra-ovarian role for steroids, particularly oestrogens and androgens, in regulating early follicular growth (Koering *et al.* 1994, Drummond *et al.* 2002, Britt *et al.* 2004, Jonard & Dewailly 2004). A key aspect to gaining greater understanding of how steroid hormones may be influencing ovarian development is first to determine which ovarian cell types have receptors to steroids. Thus, the objectives of this experiment were to identify the cell types capable of responding to oestrogen, androgens and

progestins during prenatal ovarian development and the early stages of follicular growth, which begins during fetal life and continues throughout the animal's life; the experiments were carried out in a species known to synthesise steroids during ovarian development, namely sheep.

Materials and Methods

Collection of tissue samples

All experiments were performed in accordance with the 1999 Animal Welfare Act Regulations of New Zealand. All animals were allowed access to pasture and water *ad libitum* and lambs were kept with their mothers until just prior to tissue collection. Romney ewes and lambs were killed by administration of a barbiturate overdose (pento-barbitone, 200 mg/kg). Sheep fetuses were recovered at 26, 28, 30, 32, 35, 40, 55 and 75 days of gestation. The fetuses were the results of matings of Romney ewes super-ovulated using pregnant mare's serum gonadotrophin (PMSG) (Folligon; Intervet, Lane Cove, New Zealand) and Ovagen (Immuno-chemicals Products Ltd, Auckland, New Zealand) as described previously (Smith *et al.* 1993) with Romney rams. At 4 or 5 days after mating, embryos were recovered and transferred into oestrous-synchronised recipient ewes (three embryos per ewe using a laparoscopic technique). Fetal gonadal-mesonephroi complexes were dissected from the recovered fetus and fixed in 4% (w/v) phosphate-buffered paraformaldehyde and embedded in paraffin wax. The sex of fetuses recovered before day 55 of gestation was determined by PCR using SRY gene-specific primers from tissue sections as described previously (Tisdall *et al.* 1999). The sex of fetuses recovered at days 55 and 75 was determined by examination of external genitalia. Ovaries were also collected from day-135 fetuses, 4-week-old lambs and adult ewes (all with corpora lutea) as a source of small growing follicles.

In situ hybridisation

Except where indicated, laboratory chemicals were obtained from BDH Chemicals New Zealand Ltd (Palmerston North, New Zealand), Invitrogen or Roche. Complementary cDNAs encoding a portion of the ovine progesterone receptor (PR; bases 62–359 of U30300; Genbank accession number), oestrogen receptor (ER α and ER β ; bases 1088–1573 of Z49257 and 1–416 of AF177936 respectively) and androgen receptor (AR; bases 2356–2899 of M21748) were generated using standard RT-PCR techniques. Sequences of resulting plasmids were confirmed prior to use for *in situ* hybridisation. *In situ* hybridisation was performed as previously described (Tisdall *et al.* 1999) with minor modifications. The ovaries from at least four animals were examined for expression of each of the receptor genes at all ages during gonadal development (days 26–75). Follicles of each listed classification were observed in at least four animals (day 135 of

fetal life to adult) for all receptor genes. Classification of follicles (types 1 to 5) was based on the system outlined previously (Lundy *et al.* 1999): type 1/1a consists of an oocyte surrounded by a single layer of flattened or mixed flattened and cuboidal cells; type 2 contain 1 to <2 layers of cuboidal granulosa cells; type 3 contain 2 to <4 layers of cuboidal granulosa cells; type 4 have >4 layers of granulosa cells and a well-defined theca but have not yet formed an antrum; type 5 have multiple layers of granulosa cells, a well-defined theca and a defined antrum. All follicles with signs of degeneration (i.e. pyknotic granulosa cells, lack of a distinct basement membrane or degenerate oocytes) were excluded from the study. Non-specific hybridisation was monitored by hybridising the sense RNA for each receptor to tissue collected from at least one animal per age group. Hybridisation was considered specific when the intensity of silver grains, as measured by visual assessment, over a cellular type was greater than that observed in the area of the slide not containing tissue. For all genes, hybridisation of the sense RNA over the tissue section was similar or lower in intensity to that observed on the areas of the slide not containing tissue of both the sense and antisense hybridised slides; and thus was considered to be non-specific (data not shown).

Immunohistochemistry

Immunohistochemistry to localise the presence of steroid receptor was used to confirm the presence of receptor protein in cellular types containing the mRNA. At least three animals at days 28–30, 35–40 and 55–75 were examined for the presence of the oestrogen and androgen receptor proteins. In addition, the ovaries from at least three animals were studied to identify the presence or absence of oestrogen, androgen and progesterone receptor protein for each follicular type. Immunohistochemistry was performed as previously described using a pressure-cooker antigen-retrieval method with minor modifications (Tisdall *et al.* 1999, Quirke *et al.* 2001). Briefly, following horseradish-peroxidase labelling of the secondary antibody (diluted 1:500; DAKO (DAKO Corporation, Carpinteria, CA, USA) swine anti-rabbit or rabbit anti-mouse purified immunoglobulin G (IgG)) with a DAKO ABC kit, staining sensitivity was increased using NEN tyramide signal amplification kit (New England Nuclear; Perkin Elmer Life Sciences, Boston, MA, USA). The chromagen was 3,3'-diaminobenzidine tetrahydrochloride (DAB) with haematoxylin counterstaining. The primary antibodies utilised in this study were mouse anti-bovine oestrogen receptor (12.5 μ g/ml; catalogue number 05–394, Upstate Biotechnology, Charlottesville, VA, USA), mouse anti-oestrogen receptor beta 1 (20 (post-natal) or 40 (fetal) μ g/ml, clone PPG5/10; catalogue number MCA1974S, Serotec Ltd, Oxford, UK), rabbit anti-human androgen receptor (5 μ g/ml; catalogue number sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse anti-progesterone receptor (20 μ g/ml, clone aPR-6;

catalogue number MA1-411, Affinity Bioreagents, Inc., Golden, CO, USA); this latter antibody detects only the B form of the PR and was only suitable for examination of lamb and adult ovaries as non-specific cytoplasmic staining was very high in fetal tissue. Antibody raised against a partially purified PR from nuclear extracts was trialled but was found to be unsuitable as it appeared to stain almost all cells in the ovary non-specifically. Modifications to the method included the use of a 1 mM EDTA buffer, pH 8.0, for antigen retrieval for the PR and the use of 0.1 M TRIS buffer for all steps except the primary antibody incubation (0.5 M TRIS buffer). Non-specific staining was determined by replacing the primary antibody with an equivalent amount of non-immune IgG and, when available, pre-adsorption of the primary antibody with the antigen used for immunisation.

Results

The patterns of expression of the mRNAs encoding the steroid receptors and the localisation of steroid receptor proteins were similar with a few minor exceptions. Thus, results for mRNA and protein expression are presented together. Overall, the immunohistochemical technique appeared fractionally more sensitive as detection of the protein was often observed at a slightly earlier stage of development than that for the mRNA encoding the respective protein. In addition, a few differences between the expression pattern of the mRNA and the respective protein were noted and these are detailed below.

ER α

Specific expression of mRNA for ER α was first observed on day 55 of fetal life (Fig. 1 and Table 1). A strong signal was observed in the surface epithelium and in cells entering the ovigerous cords. The hybridisation pattern observed on day 75 of fetal life was similar to that at day 55 with the strongest signal observed in the surface epithelium and pre-granulosa cells within the ovigerous cords closest to the ovarian surface, with a lower level of signal observed in connective tissue. Expression continued to be observed in the surface epithelium and ovarian stroma in both lamb and adult ewes. No signal above background was observed in type-1 to -3 follicles (Fig. 1 and Table 2). Specific hybridisation was observed in granulosa cells of type-4 and -5 follicles but not in oocytes or thecal cells (Fig. 1, Table 2). Expression was also observed in the corpus luteum when present.

The pattern of ER α protein localisation was similar to that observed for ER α mRNA. Before day 55 of gestation, faint immunostaining for ER α protein was occasionally observed in cells of the surface epithelium as well as in pre-granulosa and stroma cells. However, strong consistent staining was observed on days 55 and 75 (Fig. 2) of gestation. Staining intensity was strongest in the cells of the surface epithelium and pre-granulosa

cells near the surface of the ovary with a weaker signal being observed in stromal cells. ER α protein was observed in the surface epithelium of both lamb and adult ovaries (Fig. 2). Staining for ER α protein was occasionally observed in granulosa cells of type-1 to -3 follicles with most granulosa cells staining in type-4 and -5 follicles (Fig. 2). Staining in thecal cells was inconsistent. Occasionally, oocytes appeared to stain lightly for ER α but similar sporadic staining was also observed in the negative-control slides (see Fig. 4D for a non-immune mouse sera negative control). Strong staining for ER α protein was also observed in stromal cells, especially those around small follicles in the cortex of the ovary (Fig. 2) and around blood vessels (data not shown). Expression in luteal tissue was associated with the large luteal cells (Fig. 2).

ER β

While ER β mRNA was not detected in ovaries at days 26 or 28 of fetal life (data not shown), widespread expression was observed on days 30–40 (Fig. 3, Table 1). Expression was also evident after ovigerous cord formation (i.e. days 55 and 75; Table 1) and was noted within the ovigerous cords, in germ cells and possibly also in the pre-granulosa cells. Outside the ovigerous cords, expression was observed in the mesonephric-derived cell streams. Newly formed follicles expressed ER β mRNA in both granulosa cells and oocytes (Fig. 3). For all stages of follicular growth that were examined (i.e. types 1/1a to 5) ER β mRNA was present in oocytes, granulosa cells and the theca – which was first clearly identified in some type-3 follicles (Fig. 3, Table 2). Expression was observed in ovarian rete ($n = 3$, Fig. 3) and occasionally in isolated stromal cells. Consistent expression of ER β mRNA was not observed in the surface epithelium, the vascular system or luteal tissue.

Expression of ER β protein was observed in many cells in the ovary at the earliest day examined (day 28; Fig. 4). The presence of the protein continued to be widespread in the developing ovary including cells of the surface epithelium (varied numbers, often limited), central blastema and stroma; as well as in germ cells (Fig. 4). Expression was also observed in pre-granulosa cells, particularly as the ovigerous cords developed. Expression of ER β protein was observed in both the granulosa cells and oocyte of the newly formed follicles (data not shown). A limited number of cells in the surface epithelium continued to express ER β protein in both lamb and adult ovaries. Expression of ER β protein was also observed in both granulosa cells and oocytes of all types of follicles examined (Fig. 4) with weak signal observed in the theca of type-3 and greater follicles. ER β protein was also observed in stromal cells, particularly in areas containing numerous small (types 1 to 3) follicles. Some cells of the corpus luteum stained positive for ER β protein (data not shown).

Figure 1 Corresponding bright-field (A, C, E and G) and dark-field (B, D, F and H) views of ovaries from fetal (A–D), lamb (E and F) and adult (G and H) ovaries following hybridisation to ER α cRNA. (A and B) Day 40 of fetal life; specific signal for ER α mRNA is not observed. Note that the signal over areas of the slide not containing tissue is similar in intensity to areas containing tissue. (C and D) Day 75 of fetal life; signal can be observed in the surface epithelium (filled arrowhead), cells entering the ovigerous cords (filled arrow) and scattered stroma cells (open arrow) of the ovary as well as cells of the mesonephros (open arrowhead). (E and F) A 4-week-old lamb; expression can be observed in the granulosa of the type-4 (4) and -5 (5) follicle (compare signal intensity between antrum space of type-5 follicle and surrounding granulosa) as well as cells scattered throughout the stroma but was not detected in type-1/1a (scattered throughout left-hand edge of ovary) or type-2 (2) follicles. (G and H) Adult ewe; signal can be observed in the corpus luteum (filled arrow) with a less intense signal also observed in the stroma tissue surrounding the corpus luteum. Scale bars: approximately 50 μ m for A and B, and 100 μ m for C–H.

Table 1 Summary of expression of ER α and ER β , AR and PR mRNA and protein in the developing ovine ovary.

Gestational age (days)	ER α		ER β		AR		PR mRNA
	mRNA	Protein	mRNA	Protein	mRNA	Protein	
26	–	ND	–	ND	–	ND	–
28	–	+/-	–	+	–	+/-	–
30	–	+/-	+	+	–	+/-	–
32	–	ND	+	ND	–	ND	–
35	–	+/-	+	+	–	+/-	–
40	–	+/-	+	+	–	+/-	–
55	+	+	+	+	+	+	–
75	+	+	+	+	+	+	–

+/- faint signal observed in some animals; ND not determined.

AR

Consistent expression of mRNA encoding AR was first observed on day 55 of gestation when a faint signal was observed in the connective tissue of the ovarian medulla of all animals. By day 75, expression levels had increased with signal in connective tissue throughout the ovary, including the mesonephric-derived cell streams (Fig. 5, Table 1). However, expression was not observed inside the ovigerous cords. Expression of AR mRNA was not observed in type-1 and -2 follicles (Fig. 5) but was observed in the granulosa cells of many type-3 follicles. Strong expression of AR was also observed in the stroma, particularly around small growing follicles and thus it was difficult to determine if theca cells of type-3 follicles expressed mRNA encoding AR or if the signal observed was associated with the surrounding stroma. Both thecal and granulosa cells expressed mRNA encoding AR in type-4 and -5 follicles. Expression was also observed in luteal tissue ($n = 5$) and in scattered cells of the surface epithelium, albeit weakly.

Before day 55 of gestation, AR protein was not consistently observed in the gonad. From day 55 onwards, consistent AR immunostaining was observed in the ovarian surface epithelium and stroma (Fig. 6, Table 1). AR protein was also identified in many oocytes of follicles at all stages of development. However, staining was also observed in the oocytes of some follicles following incubation with non-immune sera. Thus, it was not possible to demonstrate unequivocally the presence of AR protein in oocytes. Granulosa cells of type-2 to -5 follicles and theca

of type-3 to -5 follicles were positive for AR protein. AR protein was also localised in some cells of the surface epithelium, most stromal cells and also luteal cells.

PR

Expression of PR mRNA was not observed in the developing ovary between days 26 and 75 of fetal life (Table 1). During follicular development, expression was observed in the theca of type-3 to -5 follicles. In addition, the granulosa cells of some type-4 and -5 follicles expressed PR mRNA faintly (Fig. 7, Table 2). Some cells of both the ovarian stroma and surface epithelium expressed PR mRNA and a weak signal was observed in luteal tissue ($n = 3$; data not shown).

Some cells of the surface epithelium and isolated stromal cells of neonatal and adult sheep did contain PR protein (Fig. 8). During follicular growth, PR protein was not consistently observed until the type-3 stage of development and was restricted to theca cells even though granulosa cells as well as thecal cells had been shown to express PR mRNA (Fig. 8, Table 2). In addition, no staining was observed in luteal tissue while faint signal for PR mRNA was observed in this tissue. It is important to note that the immunohistochemistry methodology for the detection of PR resulted in a high, non-specific staining in the cytoplasm of many cells of the ovary. This non-specific staining complicates detection of specific staining and could mask specific staining for the receptor in the nucleus of the cell. This may account for the discrepancy between the *in situ* hybridisation results and the immunohistochemistry results.

Discussion

During development of the ovary, it is clear that many cell types in the ovine ovary have the potential to respond to oestrogens and also to androgens, but it appears unlikely that they would respond to progesterone. In the sheep ovary, steroids are first produced at about day 35 of fetal life, which is around the time of morphological sexual differentiation (Lun *et al.* 1998, Quirke *et al.* 2001). At this time, oestradiol can clearly be detected in the developing ovary. The cells producing the oestradiol were

Table 2 Summary of expression of ER α and ER β , AR and PR mRNA and protein in small growing follicles.

Follicular type	ER α		ER β		AR		PR	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
1, 1a	–	+/- gc	o, gc	o, gc	–	o?	–	–
2	–	+/- gc	o, gc	o, gc	–	o?, gc	–	–
3	–	+/- gc	o, gc, t	o, gc, t	gc, t	o?, gc, t	t	–
4	gc	gc	o, gc, t	o, gc, t	gc, t	o?, gc, t	t, gc*	t
5	gc	gc	o, gc, t	o, gc, t	gc, t	o?, gc, t	t, gc*	t

O, oocyte; gc, granulosa cell, t, theca (first identifiable in type 3 follicles). ? indicates expression was equivocal; *, faint signal was observed in the granulosa cells of some follicles; +/- gc denotes expression observed in a few granulosa cells.

Figure 2 Ontogeny of ER α in ovine ovaries. (A) Day-35 ovary (scale bar, 50 μ m), showing punctuate background staining. This signal was classified as background staining since the signal was not observed in all animals, was not evenly distributed across the nucleus as was observed at all later ages, and was observed overlaying part of the nucleus and part of the cytoplasm of a cell on multiple occasions. Black arrows show non-staining oogonia. (B) Day-75 ovary (scale bar, 100 μ m), with staining mainly restricted to the surface epithelium (black arrowhead) and pre-granulosa cells within the ovigerous cords (black arrow); however, some lighter staining is also evident in some cells of the stroma located between the ovigerous cords (yellow arrow). (C) Day-135 ovary (scale bar, 100 μ m), showing staining in the surface epithelium (black arrowhead), some stromal cells (yellow arrow) and in granulosa cells. The inset C1 (scale bar, 20 μ m), shows staining in most but not all granulosa cells of type-1/1a follicles. (D) Adult (scale bar, 100 μ m), with staining in granulosa cells of a large antral (type-5) follicle. (E) Corpus luteum (scale bar, 50 μ m), showing staining predominantly of the large luteal cells and tunica with lighter staining in some small luteal cells.

located in the region of the inner cortex and outer medulla (Quirke *et al.* 2001). While precise identification of the cell types producing the steroids was not possible, it was clear that germ cells did not express mRNAs for the steroidogenic enzymes. The only other cell type in the

inner cortex–outer medulla region that showed the morphological characteristics of steroidogenic cells were the pre-granulosa cells, indicating that these cells are perhaps producing steroids at this time (Juengel *et al.* 2002, Sawyer *et al.* 2002). Expression of ER β mRNA and protein

Figure 3 Corresponding bright-field (A and C) and dark-field (B and D) views from fetal ovaries following hybridisation to ER β cRNA. (A and B) Day 40 of fetal life; specific signal for ER β mRNA is observed in many cells of the ovary. (C and D) Day 135 of fetal life; signal can be observed in a few cells of the surface epithelium (filled arrowhead) as well as in granulosa cells and oocytes of newly formed type-1/1a follicles (scattered throughout left edge of ovary) and small growing type-2 (open arrowhead) and -3 (open arrow) follicles. Signal is also observed in the stroma cells of the ovary and the rete (filled arrow). Scale bar, approximately 100 μ m for all panels.

Figure 4 Ontogeny of ER β in ovine ovaries. (A) Day-28 ovary (scale bar, 50 μ m), showing staining of most cells of the ovary. (B) Day-75 ovary (scale bar, 100 μ m), staining observed in the surface epithelium (black arrowhead), pre-granulosa (black arrow) and germ cells (yellow arrowheads) within the ovigerous cords and some cells of the stroma (yellow arrow) located between the ovigerous cords. Inset (scale bar, 20 μ m) shows magnification of stroma-ovigerous cord border. (C) A 4-week-old lamb ovary (scale bar, 200 μ m) showing staining in: the surface epithelium (black arrowhead); in some stromal cells (yellow arrow); and in oocytes, granulosa cells and thecal cells – when present – of type-1 to -5 follicles. (D) Negative control, adjacent section to that shown in panel C, in which non-immune serum was used instead of primary antibody.

in the germ cells and pre-granulosa cells during the period of peak oestradiol production (i.e. days 35–40) is consistent with an autocrine/paracrine role for oestrogen, acting via ER β to regulate the formation of the ovigerous cords as well as in germ cell development/survival (Shemesh 1980, Dominguez *et al.* 1988, Pentikainen *et al.* 2000). Ovaries in baboon fetuses of similar developmental maturity to ovaries of sheep at day 75 of gestation have been shown to express ER α and ER β in a similar pattern as observed in the current study (Pepe *et al.* 2002). Further studies to examine the potential role of oestrogens in ovarian development in baboons showed that inhibition of oestrogen synthesis during the second half of gestation, by administration of an aromatase inhibitor, resulted in a disruption of normal follicular formation (Zachos *et al.* 2002). Furthermore, 10-week-old mice lacking aromatase have recently been shown to have fewer primordial and primary follicles than wild-type controls (Britt *et al.* 2004). Collectively, these data support a critical role for oestrogens in follicular formation.

Cells of the surface epithelium of the developing gonad also expressed ERs. In sheep, cells of the surface epithelium are mitotically very active during much of fetal life and most granulosa cells originate from the surface epithelium (Sawyer *et al.* 2002). Thus, a role for oestrogen in regulating the proliferation of these cells could be postulated. However, the role of oestrogen in regulating surface epithelial cells from adult animals is equivocal, with both stimulation and inhibition of proliferation observed (Bai *et al.* 2000, Murdoch & Van Kirk 2002, Wright *et al.* 2002). It seems likely that before day 55 of gestation, oestrogen regulation would occur primarily through the ER β localised to a limited number of cells of the surface epithelium. Between days 40 and 55 of gestation there is a

strong up-regulation of expression of ER α . This corresponds to a time when cells from the surface epithelium contribute a large number of pre-granulosa cells to the ovigerous cords. However, once differentiation of the surface epithelium cell to a pre-granulosa cell (i.e. association with the germ cell) occurs, ER α (unlike ER β) was down-regulated as expression of ER α was not observed in either the forming or newly-formed follicles.

ERs were also observed in stromal cells as well as the mesonephric-derived cell streams. Previous studies have described an association between the mesonephric-derived cell streams and the developing vasculature of the ovary (Juengel *et al.* 2002, Sawyer *et al.* 2002). In addition, abnormal ovarian morphology was observed in mice lacking the aromatase gene: these animals show a disorganisation of the stroma with an increase in collagen deposition in aging animals (Britt *et al.* 2000). Thus, it is possible that paracrine interaction occurs between oestradiol and ER α and ER β to influence the stroma and vascular network in the developing ovary.

While there is evidence to support a role for oestrogens in influencing ovarian development from early fetal life, androgens – by contrast – are unlikely to influence intra-ovarian events until later in fetal life as AR was not consistently expressed until day 55. Moreover, testosterone was not detectable in the developing ovary between days 30 and 75 of fetal life, although significant amounts of androstenedione were present at day 75 (Quirke *et al.* 2001). It is possible that biologically significant amounts of androgens were made earlier during gonadal development but were not detectable with the RIA as most if not all was metabolised to oestradiol. The strongest expression of AR mRNA occurred in stromal cells and cells migrating into the ovary from the mesonephros. Thus, it seems most

Figure 5 Corresponding bright-field (A, C, E and G) and dark-field (B, D, F and H) views of ovaries from fetal (A–D) and lamb (E–H) ovaries following hybridisation to AR cRNA. (A and B) Day 40 of fetal life; specific signal for AR mRNA in the ovary (left) is very light if present. (C and D) Day 75 of fetal life; signal can be observed in the surface epithelium (filled arrowhead) and was widely distributed in scattered stroma cells (filled arrow) throughout the ovary as well as many cells of the mesonephros (m) but is not observed inside the ovigerous cords (open arrow). (E and F) A 4-week-old lamb ovary; signal is observed in the granulosa and theca, but not oocytes, of the type-4 (4) and -5 (5) follicles with strong signal observed around the small growing follicles (filled arrow). Note that the type-1/1a and -2 follicles themselves are negative (scattered at the edge of the right side of the ovary). (G and H) Lamb ovary; expression can be observed in the granulosa (g) and theca (t) of this type-5 follicle as well as a few cells scattered throughout the stroma. Scale bars, approximately 100 μ m for panels A–F and 50 μ m for panels G and H.

likely that the most profound effects of androgens are on the ovarian stroma and vasculature mainly after day 40 of fetal life.

After follicles had formed, ER β mRNA and protein continued to be present in both the granulosa cells and oocytes of type-1/1a and small growing follicles and was also detected in the theca, which was present from the type-3 stage of growth. This confirms and extends the previous findings of ER β protein in granulosa cells of ovine pre-antral and antral follicles (Cardenas *et al.* 2001) and ER β mRNA in ovine antral follicles during the follicular phase (Jansen *et al.* 2001). In addition, similar to

previous reports (Tomanek *et al.* 1997), ER α protein was detected in some type-1 to -3 follicles with consistent detection of both mRNA and protein observed in the granulosa cells of type-4 (late pre-antral) and antral follicles. The inability to detect ER α mRNA prior to the type-4 stage of development suggests that this gene is up-regulated during the late pre-antral stage of development. Clearly, as with other species (Saunders *et al.* 2000), small non-growing and growing follicles can respond to oestrogens in sheep. However, the role of oestrogens in regulating early follicular growth has yet to be clearly defined (Palter *et al.* 2001, Fortune 2003).

Figure 6 Ontogeny of AR in ovine ovaries. (A) Day-28 ovary (scale bar, 50 μ m), showing a lack of specific staining in most all cells. (B) One of two ovaries collected on day 40 (scale bar, 50 μ m) with specific staining observed in the surface epithelium (black arrowhead). Signal is also observed in cells of the stroma but germ cells (yellow arrowhead) did not contain AR protein. (C) Day-75 ovary (scale bar, 100 μ m) showing staining in the surface epithelium (black arrowhead), in some stromal cells (yellow arrow) and in germ cells within the ovigerous cords (yellow arrowhead). The inset contains the corresponding area of the adjacent section which received primary antisera that had been pre-adsorbed with immunising peptide. (D) A 4-week-old lamb ovary (scale bar, 200 μ m) showing staining in the surface epithelium, in many stromal cells (yellow arrow) and in granulosa cells and theca cells of type-4 and -5 follicles. No expression was observed in most type-1/1a follicles but granulosa cells of type-2 follicles were positive for AR (see inset; scale bar, 20 μ m). Many oocytes immunostained for AR but staining was also sometimes observed in negative-control sections.

Similar to what has been reported in other species (Saunders *et al.* 2000, Cardenas & Pope 2002, Hampton *et al.* 2004), expression of AR protein was observed in granulosa cells of type-2 follicles with consistent expression of both mRNA and protein for AR observed in granulosa and theca of type-3 and larger follicles. Some studies have also reported expression of AR in oocytes (Cardenas & Pope 2002, Szoltys *et al.* 2003); however, our results for the presence of AR in ovine oocytes were equivocal. Recent reports have indicated a stimulatory role for androgens in regulating the growth of pre-antral and small antral follicles in monkeys, mice and humans (reviewed in Fortune 2003, Jonard & Dewailly 2004). Based on the expression pattern of ARs, our results are not inconsistent with a similar role in sheep.

It is possible that progesterone has some regulatory influences in small ovine follicles as the theca expressed PR mRNA, as did granulosa cells of some type-4 (large pre-antral) and type-5 (antral) follicles. Since PR protein was detectable in the theca, but not granulosa cells, it might be that these two cell types express different isoforms (Conneely *et al.* 2003). The antibody utilised for immunohistochemistry in the present study only detected the B form of the receptors; and thus if the granulosa cells only expressed the A form, this would explain the

apparent dichotomy between the *in situ* hybridisation and immunohistochemistry results. However, the differences between the localisation of PR mRNA and protein could also be related to differences in the sensitivities of the methodologies employed for detection. The high level of non-specific staining observed with the immunohistochemistry method may have masked low levels of specific staining for PR in some ovarian cells. Expression of PR has been observed in the theca cells of pre-antral and antral follicles, and corpora lutea of several species (Suzuki *et al.* 1994, Van den Broeck *et al.* 2002). However, expression in granulosa cells was much more variable, with PR protein being observed in bovine (Van den Broeck *et al.* 2002) but not ovine (the present study) or human granulosa cells (Suzuki *et al.* 1994).

All four steroid receptors were observed in stromal cells in neonatal and adult ovary. Expression of steroid receptors in the stroma tissue around small growing follicles has also been consistently observed in other species (Suzuki *et al.* 1994, Tomanek *et al.* 1997, Saunders *et al.* 2000, Cardenas & Pope 2002, Van den Broeck *et al.* 2002). In particular, strong expression of both forms of the ER as well as the AR was observed around clusters of small non-growing and small growing follicles. Thus, oestrogen and androgens may regulate small ovine follicles indirectly by regulating

Figure 7 Corresponding bright-field (A and C) and dark-field (B and D) views of ovaries from lambs following hybridisation to PR cRNA. (A and B) A 4-week-old lamb ovary. Signal is observed in the theca (t) of this type-5 follicle (obliquely cut) but little or no signal is observed in the granulosa cells (g). Strong signal was also observed in a group of stroma cells (filled arrow) located next to this follicle. (C and D) Lamb ovary; expression can be observed in the granulosa (g) and theca (t) of this type-5 follicle as well as in a group of stromal cells (filled arrow). Scale bar, approximately 100 μm for all panels.

the stromal cell–follicular interactions as well as through direct regulation of follicular cells. One potential mechanism by which steroids may indirectly regulate early follicular growth through interaction with the stromal cells is through regulation of vascularisation of the small growing follicle (Dubey *et al.* 2000).

In conclusion, expression of receptors for steroid hormones was observed in the developing ovary and in small growing follicles. In particular, ER β was highly

expressed in granulosa and germ cells before, during and after follicular formation. Expression of AR and ER α was also observed in some cells of the developing gonad. In addition, ER α , AR and PR were also expressed in small growing follicles in a developmental stage- and cellular type-specific manner. Thus, steroid hormones have the potential to regulate many aspects of ovarian function including development of the ovary, the formation of the follicle and early follicular growth in sheep.

Figure 8 Ontogeny of PR in ovine ovaries. (A) A 4 week-old lamb ovary (scale bar, 200 μm) showing specific staining in the surface epithelium (filled arrowhead; see inset A1 (scale bar, 20 μm) for higher magnification) and in theca cells of type-3 and greater follicles (follicle type indicated by number), as well as in a few scattered stromal cells (see inset A2 (scale bar, 20 μm) for higher magnification). (B) Adjacent section to that shown in panel A, at the same magnification, in which non-immune sera was used instead of primary antibody. Note that the background staining observed is in the cytoplasm of the cells and is not located in the nucleus, where specific staining for PR would be located.

Acknowledgements

This research was supported by the New Zealand Foundation for Research, Science and Technology. The authors would like to thank Drs Allen Garverick and Mike Smith, Department of Animal Science, University of Missouri, Columbia, Missouri for providing the ER α and PR cDNAs. The authors would also like to thank Norma Hudson, Anne O'Connell, Doug Jensen and Peter Smith for help with collection of tissues; Lee-Ann Still and Lynn O'Donovan for preparation of tissue sections; Anita Burgess and Tracy Scott for technical assistance; and Alan Barkus for help with preparation of photographs.

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Received 23 February 2005

First decision 22 April 2005

Revised manuscript received 20 May 2005

Accepted 21 July 2005